SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Sousa, Rui
Jendrisak, Jerome J.

(ii) TITLE OF INVENTION: METHODS FOR USING MUTANT RNA POLYMERASES WITH REDUCED DISCRIMINATION BETWEEN NON-CANONICAL AND CANONICAL NUCLEOSIDE TRIPHOSPHATES

(iii) NUMBER OF SEQUENCES: 5

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAGACCGG AAU 13

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGAAATTAAT ACGACTCACT ATA 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGGGGGGG GACT 14

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACACGGCG AA 12

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCGGGATGG AATGGAGTAT TCGCCGTGTC CATGGCTGTA AGTATCC 47

Appendix 1

Table I: Relative Selectivity of Y639F and W.T. Polymerase for rNTPs vs. dNTPs

	rATP/dATP	rUTP/dTTP	rCTP/dCTP	rGTP/dGTP
Y639F	8.5-10 (Mg)*	1.7-1.9	2.4-2.5	.93-1.6
	.9-1.0 (Mn)*	.48-.76	.55-.80	.98-.99
W.T.	72-83 (Mg)*	22-25	110-150	51-67
	6-14 (Mn)*	2.3-2.8	4.3-5.1	4.4-6.3

Reactions were carried out with all 4 rNTPs (.5 mM) in great excess over radioactive rNTPs or dNTPs. Relative selectivity was determined from the relative percentages of radioactive rNTP vs. dNTP incorporated into RNA. Maximal incorporation was less than ~30% of total input radioactivity with all data points used so as to limit effects due to changing NTP concentrations during the experimental time course. The numbers shown give the range for 2 experiments.

*For each rNTP/dNTP and polymerase the upper numbers are those obtained in Mg^{++} buffer, the lower numbers are from Mn^{++} buffer. Polymerases at 10^{-8} M. Template was supercoiled pT75 at 10^{-7} M.

Table II: Relative Activity of Y639F and W.T. Polymerase with Different rNTP/dNTP Mixes

	W. T. (Mg^{++})	Y639F (Mg^{++})	W.T. (Mn^{++})	Y639F (Mn^{++})
4 rNTPs	200	200	21-23	9
3 rNTPs, dTTP	11-13	90-96	7-8	4
3 rNTPs, dUTP	9-11	82-91	10-11	7-8
3 rNTPs, dATP	1-2	73	1-2	2-3
3 rNTPs, dCTP	4-9	86	15	7-11
3 rNTPs, dGTP, rGMP	< .5	95-109	1-3	1.4-2.5
3 rNTPs, dGTP	< .5	43-63	.5-2	3
2 rNTPs, dCTP, dUTP	2-6	27-29	3	4-7
2 rNTPs, dCTP, dTTP	2	30	5-7	5
2 rNTPs, dCTP, dATP	< .5	20-29	.5-.9	4-7
2 rNTPs, dTTP, dGTP, rGMP	< .5	13-15	< .5	3-6
2 rNTPs, dATP, dTTP	< .5	11-14	< .5	3-4
2 rNTPs, dCTP, dGTP, rGMP	< .5	11-14	< .5	2-5
2 rNTPs, dATP, dGTP, rGMP	< .5	5-6	< .5	1-1.5
1 rNTP, dCTP, dATP, dTTP	< .5	12-14	< .5	.7-2
1 rNTP, dCTP, dATP, dUTP	< .5	10-11	< .5	2-3
1 rNTP, dTTP, dCTP, dGTP*	< .5	10-13	< .5	2-4
1 rNTP, dTTP, dATP, dGTP*	< .5	11	< .5	1.5-2
1 rNTP, dCTP, dATP, dGTP*	< .5	3-5	< .5	1-2
4 dNTPs, rGMP	< .5	< .5	< .5	.5

*These reactions also contain rGMP. Numbers give ranges from 2 experiments. Template was supercoiled pT75 (10^{-7} M), polymerases at 10^{-8} M (in Mg^{++} buffer) or 10^{-7} M (in Mn^{++} buffer). rNTPs, rGMP, dTTP were at .5 mM; dATP, dGTP were at 1 mM; dUTP was at 2.5 mM, dCTP was at 5 mM. From top to bottom the labeling NTPs were: α-P^{32} rGTP, α-P^{32} rCTP, α-P^{32} rCTP, α-P^{32} dATP, α-P^{32} dCTP, α-P^{32} dGTP, α-P^{32} dGTP, α-P^{32} dCTP, α-P^{32} dTTP, α-P^{32} dCTP, α-P^{32} dTTP, α-P^{32} dTTP, α-P^{32} dCTP, α-P^{32} dATP, α-P^{32} dTTP, α-P^{32} dCTP, α-P^{32} dTTP, α-P^{32} dTTP, α-P^{32} rCTP, α-P^{32} dCTP.

Table III: Relative activity on poly(dI)•poly(dC)

	W.T.	Y639F	G640A	Y639A	Y639S
rGTP	1000	1000	240 (200-270)	145 (142-151)	48 (47-50)
dGTP	7.4 (5.4-12.5)	964 (684-1257)	<5	5.3 (4.8-5.5)	.6
dGTP + rGMP	25 (20-27)	1070 (816-1457)	<5	25 (17-30)	4.4

Numbers give mean and range from 3 experiments. Templates were at .2 mg/ml, polymerases at 10^{-8} M. Labeling NTPs were α-P32 rGTP, α-P32 dGTP, α-P32 rATP, α-P32 dATP, as appropriate. rNTPs or rNMPs at .5 mM; dNTPs at 1 mM.

Table IV: W.T. and Y639F activity on an RNA (poly(rC)) template

	.5 mM GTP	1 mM dGTP	1 mM dGTP+.5 mM GMP
w.t.	1000	<.5	<.5
Y639F	505 (358-733)	62 (48-80)	116 (90-148)

Numbers give mean and range from 3 experiments. Template was at .2 mg/ml, polymerases at 10^{-6} M. Labeling NTPs were α-P32 rGTP, α-P32 dGTP.

Table V: Relative rates of incorporation of complementary and non-complementary rNTPs on homopolymeric templates by w.t. and mutant polymerases

I. Template: poly(dC)					
	W.T.	Y639F	G640A	Y639A	Y639S
GTP/UTP	>2000 (Mg^{++}) 53 (Mn^{++})*	>1760 55	>1320 n.d.	>184 n.d.	>50 n.d.
GTP/CTP	400 32	550 40	508 n.d.	>184 n.d.	>50 n.d.
GTP/ATP	233 9.3	338 8	388 n.d.	>184 n.d.	>50 n.d.
II. Template: poly(dT)					
	W.T.	Y639F	G640A	Y639A	Y639S
ATP/GTP	170 50	94 27	n.d. n.d.	n.d. n.d.	n.d. n.d.
ATP/UTP	121 21	94 20	n.d. n.d.	n.d. n.d.	n.d. n.d.
ATP/CTP	>340 77	>94 60	n.d. n.d.	n.d. n.d.	n.d. n.d.

Numbers are averages from 2 experiments and reflect the ratio of the percentages of labeled rNTPs incorporated into RNA in reactions in which unlabeled complementary rNTPs were in great excess (.5 mM) to labeled complementary or non-complementary rNTPs. Templates were at .1 mg/ml. Polymerases were at 10^{-8} M in Mg^{++} buffer and 10^{-7} M in Mn^{++} buffer.

*The upper number refers to results obtained in Mg^{++} buffer, the lower number to results in Mn^{++} buffer. n.d.: G640A, Y639A, Y639S were poorly active in Mn^{++} buffer, or on poly(dT) under all conditions.

Table VI: Kinetic Constants for Y639F and the W.T. polymerase with rNTPs or dNTPs

		rATP	rTTP	dTTP	dUTP	dCTP	dGTP	dATP
Y639F:	K_m	.063-.125	.034-.094	.038-.059	.052-.092	.92-1.6	.185-.264	.20-.35
	k_{cat}	150-210 s^{-1}	180-200 s^{-1}	70-110 s^{-1}	70-130 s^{-1}	50-90 s^{-1}	30-60 s^{-1}	50-70 s^{-1}
W.T.:	K_m	.034-.068	.029-.059	.209-.262	4.4-9.0	4.3-13.5	.602-.701	2.0-5.0
	k_{cat}	190-220 s^{-1}	170-230 s^{-1}	26-29 s^{-1}	25-39 s^{-1}	9-14 s^{-1}	5-9 s^{-1}	6-9 s^{-1}

Numbers give ranges from 3 experiments. The template was supercoiled pT75 at 10^{-7} M, and polymerases were at 10^{-8} M.

Table VII: 2',3'-dideoxy NTP preferences of the Y639F mutant

ATP/ddATP	TTP/ddUTP	CTP/ddCTP	GTP/ddGTP
9.0	7.0	10.0	5.0

Numbers reflect the relative specificity (k_{cat}/K_m) for an NTP vs. the corresponding ddNTP. Relative specificities could not be evaluated for the wild-type T7 RNAP because the ddNTPs are such poor substrates, but these relative specificities appear to be at least 150-fold.

Table VIII: Activity of W.T. and Y639 mutants with NTPs containing different 2'-substituents

NTP	W.T.	Y639F	Y639M
UTP	100	95±6.7	50±1.2
2'-NH2-UTP	5.9±.27	12±.41	3.6±.19
2'-F-UTP	3.1±.14	73±2.6	23±.72
2'-dUTP	2.4±.11	46±2.4	11±.46
CTP	100	103±2.3	54±3.9
2'-NH2-CTP	34±.86	60±2.5	21±.38
2'-F-CTP	3.4±.22	63±3.1	47±.70
2'-dCTP	1.6±.16	57±1.7	32±1.1
ATP	100	96±3.0	51±1.3
2'-NH2-ATP	18±.39	21±.75	.92±.035
2'-F-ATP	6.6±.12	50±1.4	9.7±.20
2'-dATP	2.7±.28	40±1.3	3.2±.11

Activity was determined with pT75 as template but with one of the rNTPs replaced with a dNTP or a 2'-modified NTP. The labeling NTP was UTP (in reactions with 2'-modified CTPs or ATPs) or CTP (in reactions with 2'-modified UTPs). Y639F and Y639M represent enzymes with substitutions of the wild-type (W.T.) tyrosine at position 639 by phenyalanine (F) or methionine (M), respectively.

Appendix 2

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U.S. PATENTS

U.S. patent 4,683,202

U.S. patent 4,965,188

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